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Activation of Human Factor V by Meizothrombin*

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A recombinant human prothrombin was prepared in which Arg^{155} was replaced by Ala. The recombinant prothrombin was converted into a meizothrombin derivative (R155A meizothrombin) that was resistant to autocatalytic removal of the fragment 1 domain. R155A meizothrombin appeared to be a potent factor V activator in reaction mixtures that contained negatively charged phospholipid vesicles. Factor V activation by R155A meizothrombin was characterized by second-order rate constants of 0.06×10^6 M^{-1} s^{-1} in the absence of phospholipid and 18×10^6 M^{-1} s^{-1} in the presence of 60 μM phospholipid vesicles composed of a 10:90 mol/mol mixture of phosphatidylserine (PS) and phosphatidylcholine (PC). The rate constant for thrombin-catalyzed activation of factor V was hardly affected by the presence of phospholipid vesicles and was 4.0×10^6 M^{-1} s^{-1}. The initial rate of activation of 3 nM factor V by R155A meizothrombin was a function of the concentration of PS/PC vesicles present in the reaction mixture, and the calculated rate constant reached a plateau value at ≥50 μM PS/PC. Gel electrophoretic analysis of factor V activation showed that R155A meizothrombin and thrombin cleaved the susceptible peptide bonds in factor V at different rates. However, both activators finally generated a factor Va molecule composed of a heavy chain with an M_r of 104,000 and a light chain doublet with M_r values of 74,000 and 71,000. Since meizothrombin is one of the major reaction products formed during the initial phase of prothrombin activation, these findings are indicative of a significant contribution of meizothrombin to *in vivo* factor V activation.

Human blood coagulation factor V is a single chain glycoprotein (1, 2) that, during hemostasis, is converted into factor Va via limited proteolysis by enzymes (*e.g.* thrombin and factor Xa) generated after the initiation of blood coagulation (1–6). Factor Va is the nonenzymatic cofactor of the prothrombin-activating complex, which also comprises the serine protease factor Xa, calcium ions, and a procoagulant membrane surface. Depending on the reaction conditions, factor Va accelerates prothrombin activation 10^3–10^5-fold (7–9).

The important function of factor Va in hemostatic plug formation is further underscored by the observation that, during *in vitro* coagulation, factor Va is the rate-limiting factor in prothrombin activation (10) and by the fact that the activity of factor Va is efficiently regulated by activated protein C (11). Since factor V is unable to act as a cofactor in the prothrombinase complex (7), activation of factor V must be a key event in thrombin formation.

Here we report that meizothrombin, a reaction intermediate that accumulates during the initial phase of prothrombin activation (12–15), may be an important factor V activator. Meizothrombin exhibits full activity on chromogenic substrates, but compared with thrombin, it has a reduced activity on macromolecular protein substrates (12, 16). Studies on the catalytic activities of meizothrombin are hampered, however, by the fact that it is autoproteolyzed to meizothrombin(desF1)[1] (12, 17) or thrombin (17). In this paper, we describe the preparation of a recombinant human prothrombin in which Arg^{155} is replaced by Ala. The recombinant prothrombin was converted into meizothrombin, which, due to the amino acid substitution, could not be converted to meizothrombin(desF1) and which was not measurably autolyzed to thrombin. This meizothrombin derivative is a powerful factor V activator in the presence of phospholipids. Since its activity can even exceed that of thrombin, activation of factor V by meizothrombin may be of prime importance for the initiation of prothrombin activation.

EXPERIMENTAL PROCEDURES

Materials—Ovalbumin, BSA, bovine brain PS, and egg yolk PC were purchased from Sigma. DOPC and DOPS were obtained from Avanti Polar Lipids, Inc. (Pelham, AL). Small unilamellar phospholipid vesicles were prepared as described before (18). S2238 (D-Phe-pipecolyl-Arg *p*-nitroanilide) and I2581 (*N*-dansyl-(*p*-guanidino)phenylalanine piperidide hydrochloride) were supplied by Chromogenix. Materials used for protein purification were purchased from Pharmacia Biotech Inc. The adenovirus-transformed human kidney 293 cell line (ATCC CRL1573) was from Flow Laboratories, Inc. *Escherichia coli* 1061/P3 and plasmid pcDNA1 were from Invitrogen. Iscove's and Optimem1 media were from Life Technologies, Inc. Vitamin K_1 was from Hoffmann-La Roche. Human coagulation factors were prepared by earlier described procedures (14, 18). The prothrombin activator from *Echis coloratus* was purified from the crude venom (Latoxan) by gel permeation chromatography on Superdex 200 and ion-exchange chromatography on Mono Q and Mono S. The venom prothrombin activator was applied to a Mono Q column in 50 mM Tris (pH 7.9), 100 mM NaCl, 0.1 mM $CaCl_2$ and to a Mono S column in 25 mM Hepes (pH 7.7), 50 mM NaCl, 0.1 mM $CaCl_2$ and eluted from the columns at NaCl concentrations between 150 and 200 mM. Details of the purification procedure will be described elsewhere.[2]

Cloning of Prothrombin and Construction of R155A Prothrombin—Full-length prothrombin cDNA was obtained as described before (19). Mutagenesis was performed by the polymerase chain reaction overlap extension technique (20) using two overlapping oligonucleotides (5′-CGATGACTCCAGCGTCCGAAGGCTCC-3′ and 5′-GGAGCCTTCGGACGCTGGAGTCATCG-3′ (mutated sequence is underlined)), resulting in the replacement of Arg^{155} with Ala. The mutated fragment was used to substitute the wild-type *Xho*I-*Bst*EII fragment of prothrombin in pcDNA1, and the DNA sequence was confirmed for the entire fragment.

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[1] The abbreviations used are: F1, prothrombin fragment 1; BSA, bovine serum albumin; PS, phosphatidylserine; PC, phosphatidylcholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; PAGE, polyacrylamide gel electrophoresis.

[2] G. A. F. Nicolaes, G. Tans, and J. Rosing, manuscript in preparation.

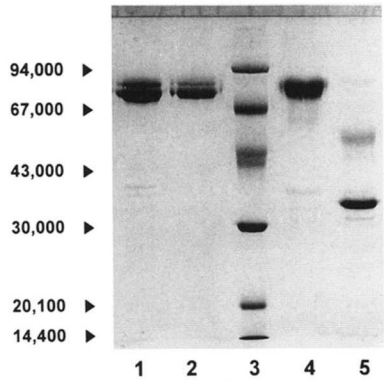

FIG. 1. **Gel electrophoretic analysis of R155A prothrombin and R155A meizothrombin.** R155A prothrombin (2.64 μM) was incubated at 37 °C with 65 ng/ml purified prothrombin activator from *E. coloratus* venom in a reaction mixture containing 25 mM Hepes (pH 7.5), 175 mM NaCl, 5 mM $CaCl_2$, and 20 μM I2581. After 4 min, the activation mixture was applied to a Mono S HR5/5 column equilibrated with 25 mM Hepes (pH 7.7) and 50 mM NaCl. R155A meizothrombin passed through this column, and I2581 was added to the meizothrombin-containing fractions to a final concentration of 20 μM. The R155A meizothrombin preparation was stored at −80 °C. The gel represents SDS-PAGE analysis of R155A prothrombin (*lane 1*), R155A meizothrombin (*lane 2*), molecular weight standards (*lane 3*), R155A prothrombin + 5% β-mercaptoethanol (*lane 4*), and R155A meizothrombin + 5% β-mercaptoethanol (*lane 5*).

Expression of Recombinant Prothrombin—The prothrombin cDNAs obtained from the pcDNA1 plasmid by digestion with *Eco*RI and *Xba*I were inserted into the *Bcl*I site of expression vector p-GTh (a gift of Dr. B. W. Grinnell, Lilly). This vector enables high expression levels in the adenovirus-transformed human kidney 293 cell line and selection of positive clones with hygromycin B since the vector contains the cistron for expression of hygromycin phosphotransferase.

Cells were grown in Optimem1 medium supplemented with 5% fetal calf serum and 10 μg/ml vitamin K_1. The cells were transfected, and colonies were selected as described by Walls *et al.* (21). The selected cell line was grown in 175-cm^2 culture dishes in the medium described above. After reaching confluency, the cells were kept serum-free in Optimem1 medium with 10 μg/ml vitamin K_1, and the medium was collected and refreshed each 72 h for 8 weeks. The medium was centrifuged for 20 min at 14,000 × *g*, and the supernatant was stored at −80 °C.

Purification and Characterization of R155A Prothrombin—In a typical purification, 0.5 liter of medium (to which 0.5 mg/ml ovalbumin was added to minimize protein losses during the first purification step) was loaded at 4 °C on a 5-ml fast flow Q-Sepharose column. The column was washed with 100 ml of 50 mM Tris (pH 7.5 at 4 °C) and 150 mM NaCl, and R155A prothrombin was eluted with a 100-ml linear gradient of 150–1000 mM NaCl in the same buffer. R155A prothrombin-containing fractions were pooled and diluted 5-fold in 50 mM Tris (pH 7.5 at 20 °C) and applied to a Mono Q column (HR 5/5). R155A prothrombin was eluted with a 30-ml linear gradient of 100–1000 mM NaCl in the same buffer. R155A prothrombin-containing fractions were pooled, dialyzed against 25 mM Hepes (pH 7.5) and 175 mM NaCl at 4 °C, and stored at −80 °C. R155A prothrombin appeared as a closely spaced doublet on nonreduced gels and as a single smeared band on reduced gels (Fig. 1). R155A prothrombin was fully carboxylated as determined by the method of Kuwada and Katayama (22). Concentrations of R155A prothrombin were determined after complete activation with *Echis carinatus* venom and by enzyme-linked immunosorbent assay using a biotinylated rabbit polyclonal antibody to human prothrombin. Antigen/activity ratios, initial rates of activation by the prothrombinase complex, and final levels of thrombin obtained were the same for R155A prothrombin and purified human plasma prothrombin.

Protein Concentrations—R155A meizothrombin concentrations were determined with S2238, considering that the amidolytic activity/mole of R155A meizothrombin is the same as that of thrombin (12, 16).

Factor Va was quantitated by determining the rate of factor Xa-catalyzed prothrombin activation in reaction mixtures that contained a limiting amount of factor Va and saturating concentrations of phospholipid vesicles (50 μM PS/PC, 10:90 mol/mol), factor Xa (5 nM), and prothrombin (0.5 μM) (18). The inhibitor I2581 (20 μM) was present to prevent further activation of factor V by thrombin generated in the assay mixture. I2581 did not interfere with the subsequent thrombin determination since it is a reversible inhibitor whose inhibitory action in the thrombin assay was essentially eliminated by dilution and by the presence of S2238. The factor Va molar concentration was calculated from the rate of prothrombin activation using a turnover number of 6000 mol of prothrombin activated per min/mol of factor Xa-Va complex (14).

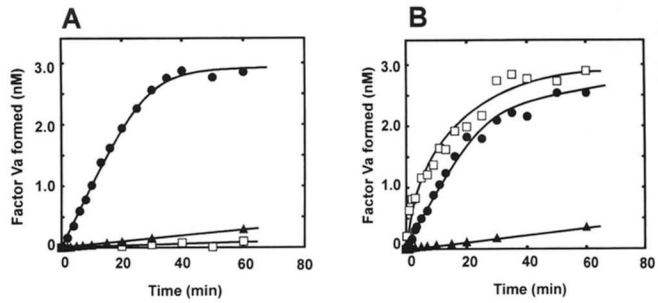

FIG. 2. **Time courses of factor V activation by thrombin, R155A meizothrombin, and meizothrombin(desF1).** Human factor V (3 nM) was activated at 37 °C with 0.15 nM thrombin (●), R155A meizothrombin (□), or meizothrombin(desF1) (▲) in the absence (*A*) or presence (*B*) of 60 μM DOPS/DOPC (10:90 mol/mol) vesicles in a reaction mixture containing 25 mM Hepes (pH 7.5), 175 mM NaCl, 5 mM $CaCl_2$, and 5 mg/ml BSA. After the time intervals indicated, aliquots from the activation mixture were assayed for the presence of factor Va as described under "Experimental Procedures."

RESULTS

Preparation of R155A Meizothrombin—R155A prothrombin was converted into R155A meizothrombin with the purified prothrombin activator from *E. coloratus* venom. R155A meizothrombin was separated from the venom activator on a Mono S column. This column material did not bind R155A meizothrombin, but retained the venom activator. Immediately after elution of R155A meizothrombin, the thrombin inhibitor I2581 (final concentration of 20 μM) was added to prevent autocatalytic peptide bond cleavages. SDS-PAGE analysis of R155A prothrombin and of the R155A meizothrombin that eluted from the Mono S column showed that R155A prothrombin was completely converted into R155A meizothrombin and that no further peptide bond cleavages had occurred. R155A meizothrombin was stored at −80 °C. Before use, I2581 was removed by passage through a prepacked Sephadex G-25 PD-10 column. The meizothrombin preparation thus obtained was diluted to 50 nM and was stable for at least 12 h at 0 °C.

Activation of Factor V by R155A Meizothrombin, Meizothrombin(desF1), and Thrombin—Time courses of factor V activation by R155A meizothrombin, meizothrombin(desF1), and thrombin are presented in Fig. 2. In free solution, R155A meizothrombin and meizothrombin(desF1) were poor factor V activators, with activities that were <5% of that of thrombin (Fig. 2*A*). However, when negatively charged phospholipid vesicles were included in the activation mixture, there was a drastic increase in the rate of R155A meizothrombin-catalyzed activation of factor V, while there was hardly any effect on factor V activation by thrombin or meizothrombin(desF1) (Fig. 2*B*). We verified that factor V activation in reaction mixtures containing phospholipids and R155A meizothrombin was not due to α-thrombin formed by autoproteolysis of R155A meizothrombin. This is concluded from the fact that R155A meizothrombin present in activation mixtures retained full amidolytic activity when incubated with antithrombin III + heparin, which shows that it is not converted into α-thrombin since this would be readily inhibited by these inhibitors (12).

Initial rates of factor Va generation were linear in time between 0 and 3 min and directly proportional to the concentrations of factor V (0–5 nM) and activator (0–0.3 nM) present in the reaction mixture (data not shown). This shows that factor V activation under these conditions is first-order in both factor V and activator (R155A meizothrombin, meizothrombin(desF1),

TABLE I
Second-order rate constants for factor V activation

Rate constants for factor V activation by thrombin meizothrombin(desF1), or R155A meizothrombin were calculated from the initial rates of factor Va formation obtained from the time courses of factor V activation presented in Fig. 2.

Factor V activator	k	
	No lipid	+60 μM DOPS/DOPC
	M^{-1} s^{-1}	
Thrombin	4.0×10^6	3.6×10^6
Meizothrombin(desF1)	0.17×10^6	0.22×10^6
R155A meizothrombin	0.06×10^6	18×10^6

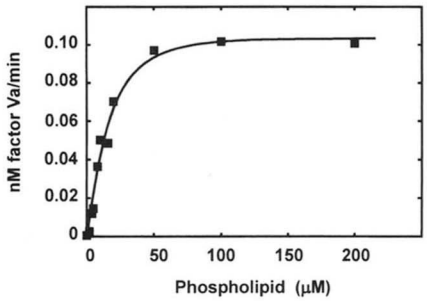

FIG. 3. **Phospholipid dependence of R155A meizothrombin-catalyzed activation of factor V.** Human factor V (3 nM) was activated at 37 °C with 0.03 nM R155A meizothrombin in a reaction mixture containing 25 mM Hepes (pH 7.5), 175 mM NaCl, 5 mM $CaCl_2$, 5 mg/ml BSA, and the indicated concentrations of DOPS/DOPC (10:90 mol/mol) vesicles. After appropriate time intervals, aliquots were taken from the activation mixture and assayed for the presence of factor Va as described under "Experimental Procedures." Initial rates of factor V activation were obtained from the time courses of factor Va formation by linear regression.

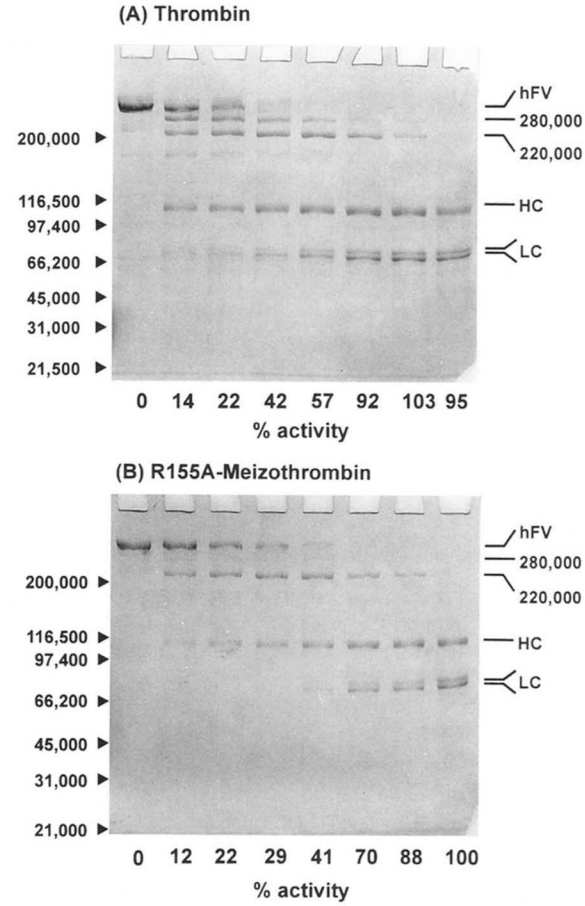

FIG. 4. **Gel electrophoretic analysis of factor V activation by thrombin and R155A meizothrombin.** Human factor V (*hFV*; 365 nM) was activated at 37 °C with 2.5 nM thrombin (*A*) or R155A meizothrombin (*B*) in a reaction mixture containing 25 mM Hepes (pH 7.5), 175 mM NaCl, 5 mM $CaCl_2$, and 60 μM DOPS/DOPC (10:90 mol/mol) vesicles. After different time intervals, aliquots from the activation mixture were assayed for the presence of factor Va as described under "Experimental Procedures." At the same times, 10-μl aliquots from the activation mixture were mixed with 10 μl of SDS- and mercaptoethanol-containing gel buffer. To allow correlation of activation with product formation, the gel represents SDS-PAGE analysis of samples that are matched as closely as possible with respect to the factor Va activities generated in the reaction mixtures. *HC* and *LC*, heavy and light chains of factor Va, respectively.

or thrombin), which allows the calculation of second-order rate constants for factor V activation from the initial rates of factor Va formation. These rate constants are summarized in Table I. Thrombin catalyzed factor V activation with second-order rate constants of 4.0×10^6 M^{-1} s^{-1} in the absence of phospholipid vesicles and 3.6×10^6 M^{-1} s^{-1} in their presence. The rate constant for meizothrombin(desF1)-catalyzed factor V activation was ~20-fold lower ($k = 0.17 \times 10^6$ M^{-1} s^{-1}) and was also hardly affected by the presence of phospholipid. In free solution, R155A meizothrombin was the weakest factor V activator ($k = 0.06 \times 10^6$ M^{-1} s^{-1}). However, the presence of 60 μM PS/PC (10:90 mol/mol) vesicles caused a 300-fold increase in the initial rate of R155A meizothrombin-catalyzed factor V activation, yielding a rate constant ($k = 18 \times 10^6$ M^{-1} s^{-1}) that even exceeds that of thrombin.

The initial rate of factor V activation by R155A meizothrombin was a function of the phospholipid concentration in the reaction mixture. The rate constant calculated from the initial phase of time courses of activation of 3 nM factor V by R155A meizothrombin increased from 0.06×10^6 M^{-1} s^{-1} in the absence of phospholipid vesicles to an average plateau value of 19×10^6 M^{-1} s^{-1} at PS/PC (10:90 mol/mol) vesicle concentrations of ≥50 μM (Fig. 3). Half-maximal rates of factor V activation were observed at ~10 μM PS/PC.

A remark is due here regarding the equation that describes time courses of factor V activation such as those presented in Fig. 2. Theoretically, the time course of product generation of an enzyme-catalyzed reaction determined under pseudo first-order conditions is described by the equation $P_t = S_0(1 - e^{-k \cdot t})$, in which P_t is the product concentration at time t, S_0 is the substrate concentration at $t = 0$, and k is the pseudo first-order rate constant of the reaction. The second-order rate constant of the reaction is obtained after dividing the pseudo first-order rate constant by the enzyme concentration. The time courses of thrombin-catalyzed factor V activation could be fitted with this equation and yielded a rate constant equal to that calculated from the initial rate of factor Va formation. However, the time course of R155A meizothrombin-catalyzed activation of factor V (Fig. 2*B*) did not adhere to the equation for a pseudo first-order reaction. The experimental curve was biphasic, with a rapid initial phase (60% of the reaction) followed by a slower second phase that completes factor V activation. Possible explanations for this biphasic behavior are given under "Discussion."

SDS-PAGE Analysis of Factor V Activation by Thrombin and R155A Meizothrombin—Generation of reaction intermediates and products during factor V activation by thrombin and R155A meizothrombin was analyzed by SDS-PAGE (Fig. 4, *A* and *B*). In agreement with the literature (1–6), thrombin-catalyzed activation of factor V proceeds via the characteristic high molecular weight intermediates of 280,000 and 220,000. The M_r 280,000 intermediate was, however, hardly formed when factor V was activated by R155A meizothrombin (Fig. 4*B*). This indicates differences in either the order or the rate of peptide bond cleavage during factor V activation by thrombin or R155A meizothrombin. Further processing of the high molecular weight intermediates resulted in both cases in the generation of activation fragments, which were hardly visible on the gel due

to poor staining with Coomassie Blue (5), and in the formation of a factor Va molecule that consisted of a heavy chain with an M_r of 104,000 and a light chain doublet with M_r values of 74,000 and 71,000.

DISCUSSION

Factor V activation is an important reaction in hemostatic plug formation. It results in the formation of factor Va, the protein cofactor that greatly accelerates prothrombin activation and that regulates the rate of thrombin formation. Factor V is rapidly activated by thrombin (1–6) and by factor Xa (6), which are the proteases that are generally considered to be the physiological activators of factor V.

In this paper, we report that factor V is also readily activated by meizothrombin, provided that negatively charged membranes are present as a catalytic surface. Meizothrombin is a reaction intermediate that accumulates in the early phase of prothrombin activation at concentrations that exceed those of thrombin (12–15). Meizothrombin is a serine protease that, compared with thrombin, was reported to exhibit greatly reduced activity toward macromolecular thrombin substrates such as fibrinogen (12, 16), platelets (16), and factor V (16). Evaluation of the catalytic activity of meizothrombin is, however, hampered by the fact that it is further processed into meizothrombin(desF1) (fast) or α-thrombin (slow) (12, 17) via an autocatalytic reaction. Removal of the F1 domain, which contains the γ-carboxyglutamic acid residues that function in the binding of meizothrombin to procoagulant membranes, may especially influence those catalytic activities of meizothrombin that may occur on a phospholipid surface, *e.g.* protein C and/or factor V activation. To enable activity studies of meizothrombin, we have prepared a recombinant human prothrombin in which Arg^{155} is replaced by Ala. The recombinant prothrombin was converted into a meizothrombin derivative (R155A meizothrombin) that is resistant to autocatalytic removal of the F1 domain. In free solution, R155A meizothrombin is a poor activator of factor V and activates factor V at a rate that is <2% of that of thrombin ($k = 0.06 \times 10^6$ *versus* 4.0×10^6 M^{-1} s^{-1}). However, when negatively charged phospholipid vesicles are included in the reaction mixture, there is a phospholipid-dependent increase in the rate of R155A meizothrombin-catalyzed factor V activation, reaching a plateau value with an average activation rate constant of $k = 19 \times 10^6$ M^{-1} s^{-1} at ≥50 μM PS/PC. Since activation of factor V by thrombin or meizothrombin(desF1) is hardly affected by the presence of phospholipid, we conclude that the stimulation in the case of R155A meizothrombin is due to the fact that both the enzyme (R155A meizothrombin) and the substrate (factor V) bind to the membrane surface. This simultaneous binding promotes the interaction between two poorly interacting components (R155A meizothrombin and factor V), which results in an increased rate of factor V activation. Such a mechanism of action of membranes has also been proposed for other phospholipid-dependent coagulation factor activations (23).

The final product of both thrombin- and R155A meizothrombin-catalyzed activation of factor V is a factor Va molecule that consists of the characteristic heavy and light chains. There are, however, some differences in the pattern of high molecular weight intermediate generation in the early phase of the activation reaction. Gel electrophoretic analysis (Fig. 4) shows that thrombin activates factor V via M_r 280,000 and 220,000 intermediates (designated A_1 and B in Ref. 1) by successive cleavage of peptide bonds 1–3 (Fig. 5). The M_r 280,000 intermediate is, however, hardly formed during factor V activation by R155A meizothrombin. This indicates that, under these conditions, there is a change in either the rate or the order of peptide bond cleavages during factor V activation. Such an effect may be specific for situations in which membrane-bound factor V is activated by a membrane-bound enzyme since a similar activation pattern (*i.e.* reduced M_r 280,000 intermediate formation) was also observed in the case of phospholipid-dependent activation of factor V by factor Xa (6). A phenomenon like this may be caused by a steric or juxtaposing effect that results from coordinate binding of substrate and enzyme to the membrane surface.

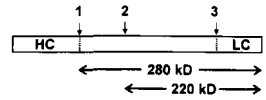

FIG. 5. **Peptide bonds cleaved by thrombin during factor V activation.** *HC* and *LC*, heavy and light chains of factor Va, respectively.

The observations that meizothrombin-catalyzed factor V activation occurs at a membrane surface via a peptide bond cleavage pattern that differs from that of factor V activation by thrombin may explain why time courses of factor Va formation are biphasic in the case of meizothrombin and monophasic in the case of thrombin. Recently, we reported that factor V preparations contain two forms of factor V with different affinities for negatively charged membranes (18). Although there may be several explanations for biphasic time courses of product generation during pseudo first-order reactions, we would like to mention two possibilities that relate to the observations mentioned above: 1) fast generation of an activation intermediate with a cofactor activity that is lower than that of a slowly formed end product (factor Va) or 2) fast activation of the factor V that binds with high affinity to phospholipid, followed by slow activation of the factor V molecules that have a low affinity for phospholipid.

The data presented in this paper show that meizothrombin is a potent factor V activator in the presence of phospholipids. The rate constant for R155A meizothrombin-catalyzed factor V activation (18×10^6 M^{-1} s^{-1}) even exceeds those determined for activation by thrombin (4×10^6 M^{-1} s^{-1}; this paper and Ref. 6) and factor Xa (3.26×10^6 M^{-1} s^{-1}; Ref. 6). Since the first prothrombin molecules that are activated during coagulation in whole blood are likely converted into meizothrombin (15), meizothrombin should be considered as a physiologically important factor V activator with a major function during the phase of coagulation in which the prothrombinase complex is generated.

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